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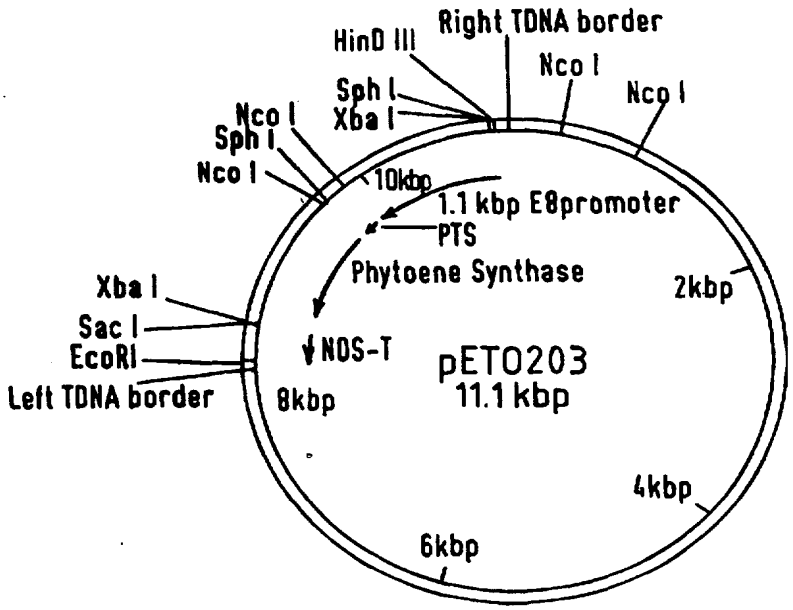
(54) Title: A METHOD FOR VISUALLY SELECTING TRANSGENIC PLANT CELLS OR TISSUES BY CAROTENOID PIGMENTATION

(57) Abstract

The present invention involves a method of visually identifying and selecting transgenic plants by carotenoid pigmentation. The method of the present invention involves culturing transgenic plant cells or tissues on a culture medium. The transgenic cells or tissues contain a recombinant chimeric DNA segment which contains at least one expression cassette. This cassette contains three components. The first component is a suitable promoter DNA segment which functions in plant cells or tissues. The second component is a DNA segment which contains a plastid targeting signal fused to the coding region of the phytoene synthase gene of the *Erwinia* group of genes which when expressed result in the production of a colored member of the biosynthetic pathway. The DNA segment causes the production of RNA which encodes a chimeric polypeptide. The third component is a 3' non-translated DNA segment. After the recombinant chimeric DNA segment is inserted into the plant cells or tissues, transgenic plant cells or tissues are identified by the appearance of orange color due to carotenoid pigmentation. The development of the carotenoid pigmentation can be used to separate transgenic plant cells from the non-transgenic plant cells and to regenerate them into plants, or to visually identify proprietary transgenic plants.



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# A METHOD FOR VISUALLY SELECTING TRANSGENIC PLANT CELLS OR TISSUES BY CAROTENOID PIGMENTATION

## FIELD OF THE INVENTION

This invention involves a method for visually identifying and selecting trangenic plant cells or tissues by carotenoid pigmentation.

## BACKGROUND OF THE INVENTION

Present systems used for the selection of transgenic plant cells involve the utilization of prokaryotic genes conferring resistance to antibiotics, herbicides, amino acids or amino acid analogs added in toxic concentrations to a regeneration medium. Although generally effective in selecting plant cells, these systems may not be effective in some types of plants, or they may result in abnormal phenotypes of the genetically engineered plants. Additionally, the use of these selective agents may have undesirable side effects and thus have raised concern about their environmental safety.

Antibiotics such as kanamycin, G418, hygromycin, bleomycin and streptomycin, among others, have been used for the selection of transgenic plants (Bevan et al., (1983), Nature 394:184-187; Dekeyser et al., (1989), Plant Physiol. 90:217-223; Hille et al., (1986), Plant Mol. Biol. 7:171-176; Jones et al., (1987), Mol. Gen. Gen. 210: 86-91; Mulsant et al., (1988), Som. Cell Mol. Gen. 14:243-252; Van den Elzen et al., (1985), Plant Mol. Biol. 5:299-302; Waldron et al., (1985), Plant Mol. Biol. 5:103-108). However, not all plants are equally sensitive to certain antibiotics. For example, kanamycin, which is used commonly, is not effective as a selective agent in gramineaceous plants; some plants from this group can tolerate up to 800 mg/L of kanamycin (Dekeyser et al.,

(1989), Plant Physiol. 90:217-223; Hauptmann et al., (1988), Plant Physiol. 86:602-606).

Herbicides such as chlorsulfuron, 2,4-D, glyphosate, phosphinotricin, and others, have been proposed as selective agents (DeBlock et al., (1987), EMBO J. 6:2513-2518; Dekeyser et al., (1989), Plant Physiol. 90:217-223; Li et al., (1992), Plant Physiol. 100:662-668; Streber, W.R.,and Willmitzer, L., (1989), Bio/Technology 7:811; Shah et al., (1986), Science 233: 478-481; White et al., (1990), Nucl. Acid Res. 18:1062). The use of a herbicide resistance trait in the identification of transgenic plants may result in increased weediness of transgenic plants because they can become herbicide-resistant weeds in the alternate years of crop rotation. Further, the use of herbicide resistant crops will increase the herbicide load in the environment.

Certain amino acids such as lysine and threonine, or the lysine derivative amino ethyl cysteine, can also be used as selective agents due to their ability to inhibit cell growth when applied at high concentrations (Shaul O. and Galili G., (1992), Plant J. 2:203-209; Perl et al., (1993), Bio/Technology 11:715-718). In this selection system expression of the selectable marker gene, which allows the transgenic cells to grow under selection, results in overproduction of these amino acids by transgenic cells. In some cases, this results in abnormal plant development (Shaul O. and Galili G., (1992), Plant J. 2:203-209). The use of visual markers for the identification of transgenic cells is an alternative to using chemically-based selective agents. Visual markers reduce the use of toxic additives in the regeneration medium. They also allow for a non-destructive, real-time assessment of transformation frequency and the dynamics of transgenic tissue regeneration (Casas et al., (1993), PNAS

90:11212-11216; Yoder et al., (1994), Euphytica 79:163-167). Visual markers based on the expression of anthocyanin, a natural plant pigment, have been proposed for use in the identification of transgenic cell lineages in both tomato and corn (Yoder et al., (1994), Euphytica 79:163-167; Pioneer Hi-Bred International, patent application WO91/02059).

Genes involved in carotenoid biosynthesis are good candidates for use as visual markers in the identification of transgenic cells. Carotenoids are a ubiquitous group of molecules that are found in many organisms. In plants, carotenoids protect cells and tissues against the deleterious effects caused by photosensitized oxidation and are used as accessory pigments in light harvesting. In mammals, carotenoids are precursors of vitamin A and are now receiving attention as one of the nutritional factors with potential anti-cancer activity. Carotenoids are also produced by some types of bacteria.

Described below is an outline of carotenoid biosynthesis in the *Erwinia* genus of bacteria.

Phytoene is the first carotenoid in the biosynthesis pathway and is produced by the dimerization of a 20-carbon atom precursor, geranylgeranyl pyrophosphate (GGPP).

The red carotenoid lycopene is the next carotenoid, and is produced from the phytoene in the pathway. Lycopene is biosynthetically synthesized from phytoene through four sequential dehydrogenation reactions by the removal of eight atoms of hydrogen. A dehydrogenase enzyme catalyzes the conversion of phytoene into lycopene. This enzyme removes four moles of hydrogen from each mole of phytoene, and is referred to as phytoene dehydrogenase-4H.

Beta-carotene is the next carotenoid produced in the carotenoid biosynthesis pathway. Beta-carotene is produced by the cyclization of unsaturated carotenoids in a

procedure not yet well understood (Bramley et al, (1988) In: Current Topics in Cellular Regulation 29:291,297). It is believed that in both plants and microorganisms a single cyclase is responsible for the conversion of lycopene to Beta-carotene.

Zeaxanthin and zeaxanthin diglucoside are the fourth and fifth carotenoids produced in the *Erwinia* genus carotenoid biosynthesis pathway. These carotenoids are useful as a food colorants, and are used as colorants in the poultry industry.

The biosynthetic pathway of the carotenoids has been elucidated based on extensive chemical evidence. The biosynthetic pathway has been described in WO91/13078 and EP 393690 A1, herein incorporated by reference. More specifically, WO91/13078 describes the characterization and expression of six genes from the Gram-negative bacteria *Erwinia herbicola*. These genes encode the enzymes geranylgeranyl pyrophosphate (GGPP) synthase, phytoene synthase, phytoene dehydrogenase - 4H, lycopene cyclase, Beta-carotene hydroxylase and zeaxanthin glycosylase. These enzymes catalyze the formation of geranylgeranyl pyrophosphate and the carotenoids phytoene, lycopene, Beta-carotene, zeaxanthin and zeaxanthin diglucoside, where each formed product (through zeaxanthin) is an immediate precursor for the next named product. European patent application 393690 describes the characterization and expression of six genes from the gram-negative bacteria *Erwinia uredovora*.

## SUMMARY OF THE INVENTION

The present invention involves a method for visually identifying and subsequently regenerating transgenic plants. The present invention also provides a method for the visual identification of proprietary transgenic

germplasm.

The method for visually identifying transgenic plant cells or tissues involves culturing non-transgenic (or non-transformed) and transgenic plant cells in a culture medium. The transgenic plant cells or tissues contain a heterologous, recombinant chimeric DNA segment which contains at least one expression cassette. An example of the plant cells or tissues that can be used in this method include but are not limited to tomato, cucurbits, pepper, lettuce and carrots.

At least one expression cassette must contain a promoter DNA segment which functions in specific plant cells to cause the production of an RNA sequence from the DNA segment described as the second component. The second component is a DNA segment which contains a plastid targeting signal fused to the amino-terminal end of the coding region of the phytoene synthase gene from the *Erwinia* group of genes, which when expressed results in the production of a carotenoid. The preferred gene is the phytoene synthase gene from *Erwinia herbicola*.

The DNA segment containing the plastid targeting signal and phytoene synthase gene produces mRNA which encodes a chimeric polypeptide. The chimeric polypeptide is produced in the cytoplasm and then transported to the plastids of the plant cells by the plastid targeting signal contained in the DNA segment.

The third component of the expression cassette is a 3' non-translated DNA segment. This segment contains sequences that in plant cells or tissues result in the termination of transcription and additional sequences that when transcribed into RNA result in the addition of a polyadenylate tract of residues to the 3' end of the RNA, which encodes the chimeric polypeptide.

The transgenic plant cells or tissues may also contain

a heterologous, recombinant chimeric DNA segment which contains additional expression cassettes. The first expression cassette is the same as the expression cassette described above. It contains a suitable promoter DNA segment, a DNA segment containing a plastid targeting signal fused to the amino-terminal end of the coding region of the phytoene synthase gene from the *Erwinia* group of genes which when expressed results in the production of a carotenoid, and a 3' non-translated termination segment.

The second and subsequent expression cassettes will each contain a promoter segment that controls the expression of a DNA segment which encodes a second gene that is expressed in the transformed plant, and a 3' non-translated segment. The second and subsequent genes may be any DNA sequence that one wishes to express in plants.

The transgenic and non-transgenic plant cells or tissues are grown for a sufficient period of time in culture to allow the transgenic plant cells or tissues to express the phytoene synthase gene, and to accumulate a colored carotenoid product. Transgenic plant cells are identified from the non-transgenic plant cells by the appearance of orange or red color due to carotenoid pigmentation. Once the transgenic plant cells or tissues are identified, the transgenic plant cells are recovered and regenerated into plants.

The recombinant chimeric DNA segment described above can be inserted into a vector for use in the method of this invention. Any vector can be used in this invention; however, the preferred vectors are those referred to as binary vectors. The DNA of interest can be delivered from the vector plasmid to the plant via *Agrobacterium*-mediated gene transfer.

In addition, the recombinant chimeric DNA segment can be introduced into the plant cells or tissues by a variety

of other techniques which are well known to those skilled in the art such as electroporation, microinjection and microprojectile bombardment.

The present invention also encompasses transgenic plants which contain the expression cassettes described above as well as seed generated from said transgenic plants.

The present invention also involves a method for the visual identification of proprietary transgenic germplasm. The method involves culturing an explant (e.g. leaf, cotyledon, root or stem fragments) on a culture medium that promotes formation of callus tissue. The proprietary transgenic plants contain a heterologous, recombinant chimeric DNA segment which contains at least one expression cassette. An example of the plants that can be used in the method include but are not limited to tomato, cucurbits, pepper, lettuce and carrots.

At least one expression cassette contains a promoter DNA segment which functions in specific plant cells or tissues to cause the production of an RNA sequence from the DNA segment described as the second component. The second component is a DNA segment which contains a plastid targeting signal fused to the amino-terminal end of the coding region of the phytoene synthase gene from the *Erwinia* group of genes, which when expressed results in the production of a carotenoid. The preferred gene is the phytoene synthase gene from *Erwinia herbicola*.

The DNA segment containing the plastid targeting signal and phytoene synthase gene produces RNA which encodes a chimeric polypeptide. The chimeric polypeptide is produced in the cytoplasm and then transported to the plastids of the plant cells by the plastid targeting signal contained in the DNA segment.

The third component of the expression cassette is a 3'

non-translated DNA segment. This segment contains sequences that in plant cells result in the termination of transcription and additional sequences that when transcribed into RNA result in the addition of a polyadenylate tract of residues to the 3' end of the RNA, which encodes the chimeric polypeptide.

The proprietary transgenic plants may also contain a heterologous, recombinant chimeric DNA segment which contains additional expression cassettes. The first expression cassette is the same as the first expression cassette described above. It contains a suitable promoter DNA segment, a DNA segment containing a plastid targeting signal fused to the amino-terminal end of the coding region of the phytoene synthase gene from the *Erwinia* group of genes which when expressed results in the production of a carotenoid, and a 3' non-translated termination segment.

The second and subsequent expression cassettes will each contain a promoter segment that controls the expression of a DNA segment, which encodes a second gene that is expressed in the transformed plant, and a 3' non-translated segment. The second and subsequent genes may be any DNA sequence that one wishes to express in plants.

To identify proprietary transgenic germplasm, the explant (e.g. leaf, cotyledon, root or stem fragments) is cultured for a sufficient period of time under conditions that allow for the creation of callus, and for the calli cells to express the phytoene synthase gene, and to accumulate a colored carotenoid product. Transgenic plants are identified by the appearance of an orange to red colored callus.

Finally, the present invention involves a plasmid designated as pETO203 having American Type Culture Collection accesion number 97282.

## DESCRIPTION OF THE DRAWINGS

Figure 1 shows an orange-pigmented tomato callus that was excised from surrounding callus using the pigmentation as guidance. The green "buds" are new meristems that are differentiating from the orange callus.

Figure 2 shows recallusing and shoot regeneration from explants derived from transgenic tomato plants expressing the phytoene synthase gene. The green explant produces orange callus from which green shoots are regenerated.

Figure 3 is a map of the plasmid vector pETO203.

## DETAILED DESCRIPTION OF THE INVENTION

The present invention uses carotenoid pigmentation of transgenic plant cells or tissues for two applications: (1) the *in vitro* visual selection of transgenic plant cells and (2) the visual identification of proprietary transgenic plants.

The first application involves a method of visually selecting transgenic plant cells or tissues from non-transgenic plant cells. The first step of the method involves culturing non-trangenic and transgenic plant cells or tissues *in vitro*. As used herein, the term "plant cells" encompasses any material from a plant which has a nucleus and cytoplasm surrounded by a membrane. It includes plants grown in a culture medium as cell suspensions, microspores, protoplasts or explants. As used herein, the term "plant tissues" encompasses a group of plant cells organized into a structural and functional unit. Also as used herein, the term "transgenic plant" refers to a plant that contains chromosomally integrated foreign or heterologous DNA. The transgenic plant cells used in this method contain a heterologous, recombinant chimeric DNA segment which contains at least one expression

cassette.

The second application involves a method of visually identifying proprietary transgenic plants. The first step of the method involves culturing explants derived from the said proprietary plants *in vitro*. As used here in the term "explants" encompasses any organ or tissue from a plant that is amenable to *in vitro* culture. Explants can be fragments of roots, stems, hypocotyls, cotyledons, leaves, petioles, petals, etc.

Proprietary transgenic plants from which the explants are taken contain a heterologous recombinant chimeric DNA segment which contains at least one expression cassette.

Heterologous refers to an expression cassette that is not present in a non-transformed cell. An expression cassette is a DNA segment that contains a gene to be expressed operatively linked to a DNA segment that contains a promoter and to a DNA termination segment, as well as any other regulatory signals needed to affect proper expression and processing of the expression product.

The expression cassette used in both applications of this invention contains a chimeric gene composed of a promoter DNA segment which functions in plant cells or tissues, a chimeric DNA segment which comprises a plastid targeting signal fused to the coding region of the phytoene synthase gene from the *Erwinia* genus group and a 3' non-translated DNA segment.

In the method for visually selecting transgenic plant cells or tissues, non-transgenic and transgenic plant cells or tissues are cultured in a suitable culture medium and allowed to grow for a sufficient period of time to allow the transgenic plant cells or tissues to express the phytoene synthase gene and accumulate a colored carotenoid product. The transgenic plant cells or tissues are then identified by their carotenoid pigmentation. Examples of

the types of plants that can be used in this method include but are not limited to: tomato, cucurbits, pepper, lettuce and carrots. Cucurbits is defined as the Cucurbitaceae family, which includes squash, melon, pumpkin, and cucumber plants.

Transgenic plants regenerated as a result of this method contain the expression cassette described above. The expression of the phytoene synthase gene in these plants and the resulting plants from it and the orange pigments of the plant cells can be used as a marker in hybrid seed production. Also protoplasts from these plants can be used to detect the protoplast fusion product based on color.

In the method for visually identifying proprietary transgenic plants, explants from proprietary transgenic plants are cultured for sufficient period of time to produce callus and to allow the callus cells to express the phytoene synthase gene. If these proprietary transgenic plants contain the expression cassette described above, then the plants can be identified by the carotenoid pigmentation of the callus. This method can be used to monitor the unauthorized use of proprietary germplasm by a competitor. Examples of plants that can be used in the method include but are not limited to: tomato, cucurbits, pepper, lettuce and carrots.

The expression cassette used in both applications of this invention contains a suitable promoter DNA segment which functions in plant cells or tissues and is operatively linked to the DNA segment. The preferred promoter is a promoter that functions during defined stages of plant regeneration *in vitro*, such as the E8 promoter from tomato disclosed in Deikman J. and Fischer, R.L., (1988) EMBO J., 7:3315-3320, and in Deikman et al., (1992) Plant Physiol. 100:2013-2017, hereby incorporated by

reference. The E8 promoter has been described as active at the onset of ripening and in unripe fruit treated with exogenous ethylene.

The use of the E8 promoter to express specific transgenes in tomato fruit is well documented (International Application No. PCT/US94/03886). Previously, it has been shown that the E8 promoter could be successfully used to express both a naturally occurring tomato gene, such as polygalacturonase (Giovannoni et al., (1989) Plant Cell 1:53-63), and a gene that is not part of the tomato chromosome, such as monellin (Penarrubia et al., (1992) Bio/Technology 10:561-564) in the fruit.

In a few of these prior art publications, researchers investigated the tissue-specific expression pattern of E8 and collectively found that this promoter is very specific in its expression pattern. More specifically, researchers have discovered that the E8 promoter drives expression during defined stages of fruit development.

In the PCT/US94/03886 application, for example, the E8 promoter was used to drive AdoMetase expression. According to the application, "Several transgenic plants were assayed for their ability to synthesize AdoMetase mRNA using a sensitive RNAase protection assay (RPA) (Example 3). Figures 6 and 7 show the results of an RPA using the fruit from two transgenic plants (ESKN and SESKN) at different stages of fruit ripening. Other tissues from these plants including immature and mature leaves flowers, and stems were negative from the presence of AdoMetase RNA" (p. 15, lines 11-19).

The inventors of this invention have found that the E8 promoter is active in undifferentiated plant callus tissue during defined stages of *in vitro* plant regeneration.

The DNA sequence of the E8 promoter that can be used in an expression cassette is disclosed in Sequence ID. NO.

1. One skilled in the art would recognize that all or part of the DNA sequence of this promoter can be used in this invention. For example, the portion of the promoter which confers a response to ethylene in both unripe and ripe fruit and which is found at base pairs 1 to 1089, in Sequence ID. NO. 1, can be removed.

One skilled in the art would recognize that promoters, other than the E8 promoter, which are expressed during plant regeneration could be used. An example of such a promoter is the wound-inducible AoPR promoter from Asparagus (Ozcan et al., (1993) Bio/Technology 11:218-221).

Additionally, one skilled in the art would recognize that either tissue-specific, organ-specific or inducible promoters can be used if the expression cassette is to be placed in proprietary germplasm, for purposes of proprietary identification. Promoters known to be either tissue-specific, organ-specific or inducible by a variety of external stimuli are well known to those skilled in the art. The following are examples of promoters that direct gene expression in an organ-specific manner: root cortex-specific TobRD2 (Mendu N. et al., (1995), Plant Physiol. 108:48); anther-specific (Riggs, D.C. and Horsch, A., (1995) Plant Physiol. 108:117); flower petal-specific Gh-2 and Gh-3 (Yamamoto, E. and Allen, R.D., (1995), Plant Physiol. 108:135); phosphate starvation-inducible, and root-specific PIG1 (Liu, C., et al., (1995), Plant Physiol. 108:112). Regulatory elements from these promoters can be adopted for the purpose of expressing the phytoene synthase gene in an organ-specific manner as a germplasm marker gene.

The expression cassette also contains a DNA segment which comprises a plastid targeting signal and a gene which results in the production of carotenoid pigmentation. Genes that can be used to produce carotenoid pigment

accumulation include any phytoene synthase gene from the *Erwinia* genus group of genes which when expressed result in the production of a carotenoid. For example, the carotenoid genes of *Erwinia herbicola*, disclosed in WO91/13078, hereby incorporated by reference, and the carotenoid genes of *Erwinia uredovora* disclosed in European Patent Application 393690, hereby incorporated by reference, can be used.

The preferred gene to be used in the expression cassette is the phytoene synthase gene from *Erwinia herbicola* which encodes the phytoene synthase enzyme. The phytoene synthase enzyme catalyzes a reaction to produce phytoene from geranylgeranyl pyrophosphate. In the carotenoid biosynthesis pathway, phytoene is a precursor of the red carotenoid lycopene. Lycopene is the carotenoid that gives tomato fruit their red color. The DNA sequence of the phytoene synthase gene of *Erwinia herbicola* that can be used in this invention is included as Sequence ID. NO. 2. The amino acid sequence of this gene is included in Sequence ID. NO. 3. However, although this invention will be described with respect to the phytoene synthase gene from Erwinia herbicola, one skilled in the art would recognize that any of the *Erwinia* genus phytoene synthase genes whose expression would result in the production of a colored member of the biosynthetic pathway can be used.

As used in this invention, the cassette containing the phytoene synthase gene is transcribed, and mRNA is produced in the nucleus. The mRNA is then translated into a chimeric polypeptide (plastid targeting signal/mature phytoene synthase) in the cytoplasm. The plastid targeting signal (also referred to as a transit peptide) allows for the chimeric polypeptide to be transported into the plastid.

It is preferred that the phytoene synthase gene encode

a chimeric polypeptide which contains a plastid targeting signal. However, when delivered to the proper location within the plastid, it is not necessary that the mature polypeptide contain the transit peptide. The plastid is the center of different enzymatic activities in the plant cell. More particularly, the plastid is the place in the plant cell where the carotenoid pigments develop. Therefore, in order to obtain the carotenoid pigmentation necessary for use in this invention, the phytoene synthase enzyme must reach the plastid. Methods for inserting a foreign protein or polypeptide into a chloroplast of a plant are disclosed in EP 189707 B1, hereby incorporated by reference.

Other genes from the *Erwinia* genus group of genes which result in the production of a colored member of the carotenoid biosynthetic pathway can be used in this invention either alone or in one or more combinations with the phytoene synthase gene. More specifically, the first expression cassette could contain a DNA segment which comprises a plastid targeting signal and the phytoene synthase gene. The second, and subsequent expression cassettes could contain other genes from the carotenoid biosynthetic pathway. The genes that could be used in these additional expression cassettes could be any gene from the *Erwinia* genus group of genes which when expressed either alone or in the presence of other carotenoid biosynthetic pathway genes result in the production of a colored carotenoid. For example, the phytoene dehydrogenase gene from *Erwinia herbicola*, which catalyzes the conversion of phytoene into the red carotenoid lycopene, could be used in combination with the phytoene synthase gene.

The DNA and amino acid sequences of a suitable plastid targeting signal that can be used in the expression

cassette are disclosed in Sequence ID. NOS. 4 and 5. One skilled in the art would recognize, however, that another plastid targeting signal or equivalent transit peptide could be used. More specifically, the transit peptide could be obtained from various sources. For example, transit peptides of a cytoplasmic precursor of a chloroplast protein or polypeptide as disclosed in EP 0189707 B1 could be used.

When the E8 promoter is used in this invention to drive the expression of the phytoene synthase gene, this gene is expressed in the callus of the transformed plant cells resulting in the expression of carotenoid pigmentation in the said callus (See Figures 1 and 2). This illustrates a tissue-specific, or callus-specific, expression pattern of this gene under the control of the E8 promoter. Once the callus differentiates into specific tissues or organs, the colored carotenoid product is not visible, presumably because the E8 promoter directs little or no expression of the phytoene synthase gene in these tissues. In tomato, visible manifestations of the E8-phytoene synthase cassette are not observed after the callus phase until the time for the ripening of the fruit. In these specific-tissue types the action of the E8-phytoene synthase cassette again becomes visible. Indeed, tomato fruit develop color prematurely as a result of expression of the E8-phytoene synthase gene expression cassette.

The expression cassette also contains a 3' non-translated termination segment that is operatively linked to the 3' end of the coding region of the phytoene synthase gene. The termination segment should have a polyadenylation signal which functions in plants to cause the addition of polyadenylate nucleotides to the 3' end of mRNA. Several termination segments useful in plants are

well known and can be used herein. One example is the 3' nontranslated region of the nopaline synthase gene (NOS-T), (Fraley et al., (1983) PNAS 80:4803-4807) used herein. The NOS-T contains a polyadenylation signal. The DNA sequence encoding NOS-T is disclosed in Sequence ID. NO. 6. Another terminator is the 3'-nontranslated region of the pea rbcS-E9 gene, which can also be used (Coruzzi et al., (1984) EMBO J. 3:1671-1679).

As stated earlier, more than one expression cassette may be present. The second and subsequent expression cassettes will each contain a DNA segment which encodes a gene that is expressed in the transformed plant. The second and subsequent expression cassettes also will each contain a suitable promoter DNA segment which drives the expression of the gene, and a 3' non-translated segment. The promoter DNA segment and 3' non-translated segment are operably linked to the DNA segment.

The promoter used in the second expression cassette may be any promoter that controls the expression of the second gene. Examples of suitable constitutive promoters that can be used include the constitutive Cauliflower Mosaic Virus (CaMV) 35S promoter, the octopine synthase promoter (P-Ocs) and the nopaline synthase promoter (P-Nos).

The gene used in the second expression cassette can be any gene desired. For example, exemplary genes include those listed in Table 1 below, whose transformations into plants have been disclosed in the patent citations shown in that Table.

TABLE 1

| Second Gene Product | Citation |
|---|---|
| HMG-CoA Reductase | U.S. 5,306,862 |
| Phosphofructokinase | U.S. 5,387,756 |
| Waxy Locus of Wheat (antisense) | U.S. 5,365,016 |
| ADP-Glucose pyrophosphorylase (antisense) | EP 0 368 506 A2 |

| | |
|---|---|
| | EP 0 455 316 A2<br>WO 92/11382 |
| Potato-alpha-amylase | EP 0 470 145 B1 |
| Sucrose phosphate synthase | EP 0 466 995 A2<br>EP 0 530 978 |
| Granule-bound starch synthase (antisense) | WO 92/11376 |
| Tomato vacuolar invertase (antisense) | WO 92/14831 |

The second expression cassette also contains a 3' non-translatable termination segment that is operatively linked to the 3' end of the second gene. The termination segment should have a polyadenylation signal which functions in plants to cause the addition of polyadenylate nucleotides to the 3' end of mRNA. Any termination segment can be used as discussed with the first expression cassette.

The recombinant chimeric DNA segment can be inserted into a vector for use in the method of this invention. The most efficient vectors for use in this invention are binary vectors. Binary vector plasmids are derived from *E. coli* and contain small portions of the tumor-inducing plasmid from *Agrobacterium tumefaciens*. The use of *Agrobacterium*-mediated gene transfer to introduce DNA into plant cells is well known in the art (Fraley et al., (1985) Bio/Technology, 3:629; and Rogers et al., (1987) Meth. Enzymol., 153:253-277).

The salient feature of the binary plasmid is that after infection by an *Agrobacterium tumefaciens* harboring the plasmid a part of the plasmid DNA is integrated into the plant chromosomal DNA. The segments that direct this insertion are referred to as the T-DNA right and left border. The right and left T-DNA borders can be as small as 25 base pairs in length.

Recent technological advances in vectors for *Agrobacterium*-mediated gene transfer have improved the

arrangement of genes and restriction sites in vectors to facilitate construction of vectors capable of expressing various genes. The vectors described by Hajdukiewicz et al., (1994) Plant Mol. Biol. 25:989-994, which have convenient multi-linker regions can be used in this invention.

One skilled in the art would recognize that if a plasmid vector is used the vector can also contain DNA sequences that encode for kanamycin or other antibiotic resistance to ensure selection of bacterial cells containing this vector. After the vector DNA is prepared, competent *E. coli* may be transformed with the vector DNA. In order to select *E. coli* that have been transformed with the vector, cells are plated onto a medium that contains an antibiotic. *E. coli* containing the vector which has a gene that confers antibiotic resistance will grow on a medium containing that antibiotic.

One skilled in the art would also recognize that other vectors could be used. The recombinant chimeric DNA segment can be introduced into monocotyledonous or dicotyledonous plant cells or tissues using other techniques such as electroporation, microprojectile bombardment, and microinjection.

Once a suitable vector has been constructed, and transformed into an appropriate *Agrobacterium* strain, plant cells or tissues can be transformed with recombinant chimeric DNA segment containing the gene cassette or cassettes of interest containing the visual selection marker gene. This involves culturing plant cells or tissues from the target plant. Generally, seeds from the plant targeted for transformation are collected, sterilized, rinsed in distilled water and then germinated on an agar surface for approximately 72 hours in the dark at approximately 25°C. The seeds are then moved to a

lighted area under approximately 80 micromol·$m^{-2}$·$s^{-1}$ PPFD at 24-26°C. Plant tissue that is to be used for transformation is prepared by removing it from the seedlings and cutting into parts suitable for transformation with a vector described above.

If the vectors are binary vectors in the appropriate strain of *Agrobacterium tumefaciens*, the excised plant parts, which are called explants, are co-cultivated with the bacteria. After the explants are co-cultivated with the *Agrobacterium tumefaciens* harboring the binary plasmid, they are transferred to a regeneration medium that is supplemented with an appropriate antibiotic, such as carbenicillin, to eliminate the bacteria. After a period of approximately two to four weeks, explants are moved to a fresh medium. Approximately one to two weeks after the transfer, orange or red pigmented sectors become visible on the callusing edges of the explants. Once the transgenic plant cells or tissues have been identified and separated from the non-transgenic cells or tissues and calli, they are regenerated into plants.

Transgenic plant cells or tissues can be visually selected using the method and vectors described above. The method of this invention allows for the visual selection of transgenic plant cells or tissuesand regeneration of transgenic plants without incorporating antibiotic or herbicide resistance genes into the plant genome. Toxic additives such as herbicides, amino acids or amino acid analogs are not used during plant culture. Antibiotics are used only for a brief period to eliminate the *Agrobacterium* during the regeneration process.

The inventors have also found that transgenic plants containing the phytoene synthase gene develop fruit color earlier and taste different than non-transgenic fruit.

By way of example, and not limitation, an example of

the present invention will now be given.

EXAMPLE

**Construction of a binary vector**

This example describes how to prepare a binary vector referred to as pETO203. pETO203 is a binary vector that contains the 1.1 kilobase pair (kbp) E8 promoter, a DNA segment containing a plastid targeting signal fused to the coding region of a phytoene synthase gene and a 3' non-translated region that supplied a transcription termination signal. Those skilled in the art will recognize that alternative cloning strategies to those described herein can be used to clone these genetic elements into a binary vector. For a general molecular biology technique reference, refer to Sambrook et al., (1989) Molecular Cloning: A Laboratory Manual. pETO203 has been deposited with the American Type Culture Collection (ATCC), 12301 Parklawn Drive, Rockville, Maryland, 20852. pETO203 was deposited with the ATCC on September 15, 1995, and assigned ATCC Number 97282.

**Cloning of the tomato E8 promoter**

The cloning of the E8 promoter from tomato (*Lycopersicon esculentum*) has been reported using standard molecular biology techniques (Lincoln and Fischer, (1987) PNAS 84:2793-2797). The nucleotide sequence of various portions of the E8 promoter has also been reported (Deikman and Fischer, (1988) EMBO J. 7:3315-3320; and Deikman et al., (1992) Plant Physiol. 100:2013-2017)

The E8 promoter was cloned from *L. esculentum*, variety VFNT Cherry using the polymerase chain reaction (PCR). The template for the PCR reaction was genomic VFNT Cherry DNA, and synthesis was primed by two synthetic oligonucleotides. The primers were designed from the published E8 promoter

sequences. The sequence of these oligonucleotides, named E8 primers #1 and #2, are shown below:

E8 primer #1: 5' NNG AAT TCA TTT TTG ACA TC 3' (SEQUENCE ID. NO. 7)

E8 primer #2 5' GCT TTC CAT GGT CTT TTG CA 3' (SEQUENCE ID. NO. 8)

E8 primer #1 anneals to the 5' end of the reported sequence of the E8 promoter. The first two residues are indicated with an N, which represents any nucleotide. Thus, the E8 primer #1 represents a population of primers with varying nucleotide residues in the first and second positions. Following these two variable residues, the next six residues specify the recognition site for the restriction enzyme EcoRI. E8 primer #2 anneals to an area of the tomato genome that surrounds the initiation codon of the E8 gene. Two mismatches occur between the authentic E8 sequence and the E8 primer #2. As a consequence of these mismatches, PCR amplification of this region of the tomato genomic DNA using E8 primers 1 and 2 resulted in the incorporation of an NcoI restriction site surrounding the initiation codon. Digestion of the amplification product with the restriction enzymes EcoRI and NcoI facilitate the directional cloning of the E8 promoter into a binary vector with other genetic elements described below.

Plant plastids are organelles that perform many functions. Plastids have their own small genome and the capacity to produce some of their own proteins. However, most of the plastid proteins are produced in the cytoplasm, and are encoded for by nuclear genes. The proteins are synthesized with amino terminal extensions, which direct the precursor protein to the plastid. During import into the plastid, the amino terminal extension, referred to as the targeting signal or plastid targeting signal (PTS), is removed by enzymatic cleavage. In plant, plastids can differentiate into specialized organelles such as

chloroplasts and chromoplasts. Alternatively, plastids can also remain in an undifferentiated state, as they do in callus tissue.

To target the *Erwinia herbicola* phytoene synthase gene product to the plastid, a PTS from the small subunit of the ribulose-1,5-bisphosphate carboxylase oxygenase (RUBISCO) gene can be fused to the phytoene synthase gene. In a similar fashion to the cloning of the E8 promoter, PCR amplification and subsequent cloning of the RUBISCO PTS can be performed with primers containing small mismatches to the template that result in the incorporation of restriction sites at the termini of the PTS PCR product. For the purpose of illustration, primers can be designed to incorporate an NcoI site near the 5' terminus of the PTS, and an SphI site near the 3' terminus of the PTS.

**Cloning of the *Erwinia herbicola* phytoene synthase gene**

The sequence of genes involved in carotenoid biosynthesis from *E. herbicola* has been reported (Genbank Accession M87280). In a similar manner to the cloning of the E8 promoter, the *E. herbicola* phytoene synthase gene (crtB) can be cloned by PCR using *E. herbicola* DNA as a template and primers designed from the reported sequence. The incorporation of small mismatches between the primers and template that create restriction sites near the termini of the PCR product is a cloning strategy that can facilitate the cloning of the PCR amplified crtB gene. For the purpose of illustration, primers can be designed to incorporate an SphI site near the 5' terminus of the gene, and an SacI site near the 3' terminus of the gene.

**Cloning of a termination signal**

The termination signal from the nopaline synthase gene (NOS-T) can be cloned from the commercially available binary vector pBI121 (Clontech Co., Palo Alto, California). This segment contains sequences that in plant cells result

in the termination of transcription and additional sequences that when transcribed into RNA result in the addition of a polyadenylate tract of residues to the 3' end of the RNA, which encodes the chimeric polypeptide. The DNA fragment containing this genetic element can be obtained by digestion with the restriction enzymes SacI and EcoRI, followed by gel-purification of the approximately 300 base pair fragment.

**Cloning of genetic elements into a binary vector**

Binary vectors are a preferred way of delivering transgenic gene cassettes into plant chromosomes, via *Agrobacterium*-mediated transformation. Plasmid pETO183 is a preferred parent binary vector which contains a polylinker between the T-DNA borders. Alternative binary vectors can be substituted for pETO183. In the cloning strategy described herein, other binary vectors such as pBIN19 (Bevan, M. (1984) Nucl. Acids Res. 12: 8711-8721), pPZP100 and pPZP200 (Hajdukiewicz et al., (1994) Plant Mol. Biol. 25: 989-994) can be substituted for pETO183 and serve as parent binary vectors.

To produce a derivative vector of pETO183 that would contain, in this order, the 1.1 kbp E8 promoter, the PTS, the crtB gene and nopaline synthase termination signals, a ligation reaction can be prepared and would include the following DNA fragments:

1. Parent Vector: Plasmid pETO183 (other plasmids that can substituted for pET0183 include BIN19, which is commercially available from Clontech Labs, Palo Alto, California, pPZP100 and pPZP200, which are both available from Dr. Pal Maliga at Waksman Institute, Rutgers University, Piscataway, N.J.) digested with the restriction enzymes XbaI and EcoRI.

2. 1.1 kbp E8 promoter: The PCR-generated 2.2 kbp E8 promoter digested with XbaI and NcoI, and the 1.1 kbp

fragment gel-purified.

3. Targeting signal: The PCR-generated PTS DNA digested with NcoI and SphI.

4. Phytoene synthase gene: The PCR-generated DNA containing the phytoene synthase gene digested with SphI and SacI.

5. NOS-T: pBI121 digested with SacI and EcoRI and the approximately 300 base pair fragment gel-purified.

After the ligation reaction, an aliquot can be taken and used to transform *E. coli*. Transformed colonies harboring the plasmid of interest can be identified by growing the culture and isolating the plasmid DNA. Several diagnostic restriction digests of these DNAs will show whether the various genetic elements have been fused together in the proper orientation. Once putative clones containing the above mentioned genetic elements (#2-5) have been identified by restriction digestion, one can precisely determine the integrity of the insertion by sequencing the inserted XbaI-EcoRI fragment.

**Plant transformation using vector pETO203**

Tomato seeds were sterilized in 20% Clorox for 20 minutes, rinsed 3 times in sterile distilled water and placed on Murashigi and Skoog medium (Gibco) solidified with 10 grams of Noble agar (Gibco) in 135 mm Phytacon™ tissue culture vessels (Sigma, St. Louis, MO). Seeds were germinated for 72 hours at 25°C in the dark, then moved to a lighted shelf under approximately 80 micromol·$m^{-2}$·$s^{-1}$ PPFD, at 24-26°C. Plant tissue used for transformation was prepared by removing cotyledons from 7-day-old seedlings and cutting them into three parts (proximal, middle and distal to the growing point). The middle and proximal parts were used for co-cultivation with *Agrobacterium*. They were placed abaxial side down on a sterile filter paper overlaying co-cultivation medium R1F supplemented

with 16 g/L glucose, and incubated in the dark for 24 hours. Next, they were incubated for 20 minutes with bacterial inoculum containing 5 x $10^8$ CFU/mL of *Agrobacterium tumefaciens*, LBA4404::pETO203, blotted dry, and cultured on the RIF co-cocultivation medium for 48 hours, at 24°C, in the dark.

Bacterial inoculum was prepared by growing *A. tumefaciens*, LBA4404::pETO203, in 25 ml of AB medium (Chilton et al., (1974) PNAS 71:3672-3676) supplemented with 50 mg/L kanamycin (K) and 25 mg/L streptomycin (St) ($AB_{K50St25}$) on a shaker at 28°C, 180 rpm, for 24 hours. Bacteria were then pelleted by centrifuging at 8000 rpm for 10 minutes in a Beckman J2-21 centrifuge using a JA-20 rotor. The bacterial pellet fraction was resuspended in a sterile MS medium to a concentration of 5 x $10^8$ CFU/mL using a spectrophotometric optical density reading at 550 nm (0.1 OD550=2x$10^8$ CFU/mL). Prior to co-cultivation, the inoculum was supplemented with Acetsyringone (3' 5'dimethoxy-4'hydroxy-acetophenone, Sigma, St. Louis, MO) to a final concentration of 600 micromolar.

**Regeneration of transformed tomato plants**

Five hundred tomato explants from an inbred line T7 were cocultivated with *Agrobacterium tumefaciens* LBA4404::pETO203 carrying a plastid targeting signal and the phytoene synthase gene from *Erwinia herbicola* driven by the 1.1 kbp E8 promoter. After two days of cocultivation, explants were transferred to a R1/2 300 regeneration medium supplemented with 300 mg/L carbenicillin to eliminate the bacteria. After two weeks of culture explants were moved to fresh R1/2 300 medium. Sectors of orange-pigmented callus became clearly visible on the callusing edges of the explants about one week after the last transfer. These pigmented sectors were cut out and subjected to a standard regeneration protocol. The remaining non-pigmented callus

was also carried through a standard regeneration protocol. The standard regeneration protocol consisted of two-to-four week culture on R 1/2 300 medium followed by four-week-culture on a hormone-free RO 300 medium. Green tomato shoots that differentiated from the calli either on the R 1/2 300 or RO 300 meidum were detached from the surrounding callus and rooted on the RO 300 medium.

At the completion of the experiment a total of 377 tomato plants were regenerated. Among them, 85 plants were regenerated from the pigmented sectors and 292 plants were regenerated from the non-pigmented callus. All regenerated plants were indexed for presence of the phytoene synthase gene. Among the 85 plants regenerated from the orange callus, 42 plants were confirmed to be transgenic. This result indicates that expression of the phytoene synthase gene can aid in identification and isolation of transgenic plants.

Regenerated transgenic plants displayed three distinctive characteristics: they produced orange-pigmented callus in an *in vitro* culture; when grown in the field, fruit from transgenic plants developed color earlier than the nontransgenic control fruit; the fruit from the transgenic plants had a distinctly different taste than control fruit from nontransgenic plants.

**Visual identification of proprietary transgenic plants.**

Seeds from transgenic inbred line T7, transformed with the phytoene synthase gene from *Erwinia herbicola* driven by the E8 promoter (T7/E8PS), as described above and from nontransgenic control T7 inbred line were surface sterilized in 20% Clorox for 20 minutes, rinsed 3 times in sterile distilled water and placed on Murashigi and Skoog medium (Gibco) solidified with 10 grams of Noble agar (Gibco) in 135 mm Phytacon™ tissue culture vessels (Sigma,

St. Louis, MO). They were germinated for 72 hours at 25°C in the dark, then moved to a lighted shelf under approximately 80 micromol$m^{-2}s^{-1}$ PPFD, at 24-26°C.

Cotyledons from seven-day-old seedlings were used for visual identification of proprietary transgenic plants. The cotyledons were cut into three parts: proximal, middle and distal to the growing point. The middle and proximal parts were placed abaxial side down on R 1/2 regeneration medium and cultured under 80 micromol$\cdot m^{-2}\cdot s^{-1}$ PPFD, 24-26°C, 16 hours photoperiod. After two weeks of culture explants were moved to fresh R 1/2 medium. About one week after the last transfer orange-pigmented callus became clearly visible on the callusing edges of explants from the T7/E8PS plants. Explants from control T7 plants produced white and/or green callus that is typically observed during *in vitro* regeneration of tomato plants.

The presence of orange pigmented callus allowed for rapid and unequivocal identification of explants from T7/E8PS plants transformed with the phytoene synthase gene.

**Media Used**

All media used consisted of Murashigi and Skoog (Murashigi T., Skoog F., (1962) Physiol. Plant 15:437-498) salts and RO vitamins (composition listed below), which were adjusted to pH=5.7 and solidified with 9 g/L of Noble Agar (Gibco). Medium R1F was supplemented with 1 mg/L indoleacetic acid (IAA), 0.65 mg/L zeatine and 16 g/L glucose. Medium R1/2 was supplemented with 0.5 mg/L IAA, 0.325 mg/L of zeatine and 16 g/L glucose, medium RO was supplemented with 16 g/L glucose.

Table 1.
Composition of media used in tomato regeneration.

| MS Salts | mg/L |
|---|---|
| Ammonium nitrate | 1650.000 |
| Boric Acid | 6.200 |
| Calcium chloride | 440.000 |

| | |
|---|---|
| Cobaltous chloride | 0.025 |
| Cupric sulfate pentahydrate | 0.025 |
| Ferrous sulfate septahydrate | 27.800 |
| Magnesium sulfate septahydrate | 370.000 |
| Manganese sulfate monohydrate | 15.600 |
| Potassium iodide | 0.083 |
| Potassium nitrate | 1900.000 |
| Potassium phosphate monobasic | 170.000 |
| Sodium ethylenediamine tetraacetate | 37.300 |
| Sodium molybdate dihydrate | 0.250 |
| Zinc sulfate septahydrate | 8.600 |
| RO Vitamins | mg/L |
| Nicotinic acid | 5.000 |
| Thiamine HCl | 0.500 |
| Pyridoxine | 0.500 |
| Myo-inositol | 100.000 |
| Glycine | 2.000 |

Although the invention has been described primarily in connection with the special and preferred embodiments, it will be understood that it is capable of modification without departing from the scope of the invention. The following claims are intended to cover all variations, uses or adaptions of the invention, following, in general, the principles thereof and including such departures from the presented disclosure as come within known or customary practice in the field to which the invention pertains, or as are obvious to persons skilled in the field.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT: Trulson, Anna J.
Braun III, Carl J.

ii) TITLE OF INVENTION: A Method for Visually Selecting Transformed Plants Cells or Tissues by Carotenoid Pigmentation

(iii) NUMBER OF SEQUENCES: 8

(iv) CORRESPONDENCE ADDRESS:
(A) ADDRESSEE: Greer, Burns & Crain, Ltd.
(B) STREET: 233 South Wacker Drive, Suite 8660, Sears Tower
(C) CITY: Chicago
(D) STATE: IL
(E) COUNTRY: USA
(F) ZIP: 60606

(v) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: PatentIn Release #1.0, Version #1.30

(vi) CURRENT APPLICATION DATA:
(A) APPLICATION NUMBER: US
(B) FILING DATE:
(C) CLASSIFICATION:

(viii) ATTORNEY/AGENT INFORMATION:
(A) NAME: Mueller, Lisa V.
(B) REGISTRATION NUMBER: 38,978
(C) REFERENCE/DOCKET NUMBER: 1605.60833

(ix) TELECOMMUNICATION INFORMATION:
(A) TELEPHONE: 312-993-0080
(B) TELEFAX: 312-993-0633

(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 2208 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: unknown
(D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

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TCTACAAATA AGAGCAAATT TATTTATTTT TTAATCCAAT AAATATTAAT GGAGGACAAA 180

TTCAATTCAC TTGGTTGTAA AATAAACTTA AACCAATAAC CAAAGANCTA ATAAATCTGA 240

AGTGGAATTA TTAAGGATAA TGTACATAGA CAATGAAGAA ATAATAGGTT CGATGAATTA 300

ATAATAATTA AGGATGTTAC AATCATCATG TGCCAAGTAT ATACACAATA TTCTATGGGA 360

TTTATAATTT CGTTACTTCA CTTAACTTTT GCGTAAATAA AACGAATTAT CTGATATTTT 420

ATAATAAAAC AGTTAATTAA GAACCATCAT TTTTAACAAC ATAGATATAT TATTTCTAAT 480

AGTTTAATGA TACTTTTAAA TCTTTTAAAT TTTATGTTTC TTTTAGAAAA TAAAAATTCA 540

AAAAAATTAA ATATATTTAC AAAAACTACA ATCAAACACA ACTTCATATA TTAAAAGCAA 600

AATATATTTT GAAAATTTCA AGTGTCCTAA CAAATAAGAC AAGAGGAAAA TGTACGATGA 660

GAGACATAAA GAGAACTAAT AATTGAGGAG TCCTATAATA TATAATAAAG TTTATTAGTA 720

AACTTAATTA TTAAGGACTC CTAAAATATA TGATAGGAGA AAATGAATGG TGAGAGATAT 780

TGGAAAACTT AATAATTAAG GATNTTAAAA TATATGGTAA AAGATAGGCA AAGTATCCAT 840

TATCCCCTTT TAACTTGAAG TCTACCTAGG CGCATGTGAA AGGTTGATTT TTTGTCACGT 900

CATATAGCTA TAACGTAAAA AAAGAAAGTA AAATTTTTAA TTTTTTTTAA TATATGACAT 960

ATTTTAAACG AAATATAGGA CAAAATGTAA ATGAATAGTA AAGGAAACAA AGATTAATAC 1020

TTACTTTGTA AGAATTTAAG ATAAATTTAA AATTTAATAG ATCAACTTTA CGTCTAGAAA 1080

GACCCATATC TAGAAGGAAT TTCACGAAAT CGGCCCTTAT TCAAAAATAA CTTTTAAATA 1140

ATGAATTTTA AATTTTAAGA AATAATATCC AATGAATAAA TGACATGTAG CATTTTACCT

1200

AAATATTTCA ACTATTTTAA TCCAATATTA ATTTGTTTTA TTCCCAACAA TAGAAAGTCT
1260

TGTGCAGACA TTTAATCTGA CTTTTCCAGT ACTAAATATT AATTTTCTGA AGATTTTCGG
1320

GTTTAGTCCA CAAGTTTTAG TGAGAAGTTT TGCTCAAAAT TTTAGGTGAG AAGGTTTGAT
1380

ATTTATCTTT TGTTAAATTA ATTTATCTAG GTGACTATTA TTTATTTAAG TAGAAATTCA
1440

TATCATTACT TTTGCCAACT TGTAGTCATA ATAGGAGTAG GTGTATATGA TGAAGGAATA
1500

AACAAGTTCA GTGAAGTGAT TAAAATAAAA TATAATTTAG GTGTACATCA AATAAAAACC
1560

TTAAAGTTTA GAAAGGCACC GAATAATTTT GCATAGAAGA TATTAGTAAA TTTATAAAAA
1620

TAAAAGAAAT GTAGTTGTCA AGTTGTCTTC TTTTTTTTGG ATAAAAATAG CAGTTGGCTT
1680

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1740

TTGATTCATG TATGAATACT AAATAATTTT TTAGGACTGA CTCAAATATT TTTATATTAT
1800

CATAGTAATA TTTATCTAAT TTTTAGGACC ACTTATTACT AAATAATAAA TTAACTACTA
1860

CTATATTATT GTTGTGAAAC AACAACGTTT TGGTTGTTAT GATGAAACGT ACACTATATC
1920

AGTATGAAAA ATTCAAAACG ATTAGTATAA ATTATATTGA AAATTTGATA TTTTTCTATT
1980

CTTAATCAGA CGTATTGGGT TTCATATTTT AAAAAGGGAC TAAACTTAGA AGAGAAGTTT
2040

GTTTGAAACT ACTTTTGTCT CTTTCTTGTT CCCATTTCTC TCTTAGATTT CAAAAAGTGA
2100

ACTACTTTAT CTCTTTCTTT GTTCACATTT TATTTTATTC TATTATAAAT ATGGCATCCT
2160

CATATTGAGA TTTTTAGAAA TTATTCTAAT CATTCACAGT GCAAAAGA
2208

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 930 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: unknown
(D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: DNA (genomic)

(ix) FEATURE:
(A) NAME/KEY: CDS
(B) LOCATION: 1..933

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

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Met Arg Gln Pro Pro Leu Leu Asp His Ala Thr Gln Thr Met Ala Asn
 1               5                  10                  15

GGC TCG AAA AGT TTT GCC ACC GCT GCG AAG CTG TTC GAC CCG GCC ACC
                                                              96
Gly Ser Lys Ser Phe Ala Thr Ala Ala Lys Leu Phe Asp Pro Ala Thr
            20                  25                  30

CGC CGT AGC GTG CTG ATG CTC TAC ACC TGG TGC CGC CAC TGC GAT GAC
                                                             144
Arg Arg Ser Val Leu Met Leu Tyr Thr Trp Cys Arg His Cys Asp Asp
        35                  40                  45

GTC ATT GAC GAC CAG ACC CAC GGC TTC GCC AGC GAG GCC GCG GCG GAG
                                                             192
Val Ile Asp Asp Gln Thr His Gly Phe Ala Ser Glu Ala Ala Ala Glu
    50                  55                  60

GAG GAG GCC ACC CAG CGC CTG GCC CGG CTG CGC ACG CTG ACC CTG GCG
                                                             240
Glu Glu Ala Thr Gln Arg Leu Ala Arg Leu Arg Thr Leu Thr Leu Ala
65                  70                  75                  80

GCG TTT GAA GGG GCC GAG ATG CAG GAT CCG GCC TTC GCT GCC TTT CAG
                                                             288
Ala Phe Glu Gly Ala Glu Met Gln Asp Pro Ala Phe Ala Ala Phe Gln
                85                  90                  95

GAG GTG GCG CTG ACC CAC GGT ATT ACG CCC CGC ATG GCG CTC GAT CAC
                                                             336
Glu Val Ala Leu Thr His Gly Ile Thr Pro Arg Met Ala Leu Asp His
            100                 105                 110

CTC GAC GGC TTT GCG ATG GAC GTG GCT CAG ACC CGC TAT GTC ACC TTT
                                                             384
Leu Asp Gly Phe Ala Met Asp Val Ala Gln Thr Arg Tyr Val Thr Phe
        115                 120                 125

GAG GAT ACG CTG CGC TAC TGC TAT CAC GTG GCG GGC GTG GTG GGT CTG
                                                             432
Glu Asp Thr Leu Arg Tyr Cys Tyr His Val Ala Gly Val Val Gly Leu
    130                 135                 140

ATG ATG GCC AGG GTG ATG GGC GTG CGG GAT GAG CGG GTG CTG GAT CGC
                                                             480
Met Met Ala Arg Val Met Gly Val Arg Asp Glu Arg Val Leu Asp Arg
145                 150                 155                 160
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GCC TGC GAT CTG GGG CTG GCC TTC CAG CTG ACG AAT ATC GCC CGG GAT
                                                              528
Ala Cys Asp Leu Gly Leu Ala Phe Gln Leu Thr Asn Ile Ala Arg Asp
                  165                 170                 175

ATT ATT GAC GAT GCG GCT ATT GAC CGC TGC TAT CTG CCC GCC GAG TGG
                                                              576
Ile Ile Asp Asp Ala Ala Ile Asp Arg Cys Tyr Leu Pro Ala Glu Trp
              180                 185                 190

CTG CAG GAT GCC GGG CTG ACC CCG GAG AAC TAT GCC GCG CGG GAG AAT
                                                              624
Leu Gln Asp Ala Gly Leu Thr Pro Glu Asn Tyr Ala Ala Arg Glu Asn
          195                 200                 205

CGC CCC GCG CTG GCG CGG GTG GCG GAG CGG CTT ATT GAT GCC GCA GAG
                                                              672
Arg Pro Ala Leu Ala Arg Val Ala Glu Arg Leu Ile Asp Ala Ala Glu
      210                 215                 220

CCG TAC TAC ATC TCC TCC CAG GCC GGG CTA CAC GAT CTG CCG CCG CGC
                                                              720
Pro Tyr Tyr Ile Ser Ser Gln Ala Gly Leu His Asp Leu Pro Pro Arg
225                 230                 235                 240

TGC GCC TGG GCG ATC GCC ACC GCC CGC AGC GTC TAC CGG GAG ATC GGT
                                                              768
Cys Ala Trp Ala Ile Ala Thr Ala Arg Ser Val Tyr Arg Glu Ile Gly
                  245                 250                 255

ATT AAG GTA AAA GCG GCG GGA GGC AGC GCC TGG GAT CGC CGC CAG CAC
                                                              816
Ile Lys Val Lys Ala Ala Gly Gly Ser Ala Trp Asp Arg Arg Gln His
              260                 265                 270

ACC AGC AAA GGT GAA AAA ATT GCC ATG CTG ATG GCG GCA CCG GGG CAG
                                                              864
Thr Ser Lys Gly Glu Lys Ile Ala Met Leu Met Ala Ala Pro Gly Gln
          275                 280                 285

GTT ATT CGG GCG AAG ACG ACG AGG GTG ACG CCG CGT CCG GCC GGT CTT
                                                              912
Val Ile Arg Ala Lys Thr Thr Arg Val Thr Pro Arg Pro Ala Gly Leu
      290                 295                 300

TGG CAG CGT CCC GTT TAG
                      930
Trp Gln Arg Pro Val  *
305                 310
```

(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 311 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

```
Met Arg Gln Pro Pro Leu Leu Asp His Ala Thr Gln Thr Met Ala Asn
 1               5                  10                  15

Gly Ser Lys Ser Phe Ala Thr Ala Ala Lys Leu Phe Asp Pro Ala Thr
            20                  25                  30

Arg Arg Ser Val Leu Met Leu Tyr Thr Trp Cys Arg His Cys Asp Asp
        35                  40                  45

Val Ile Asp Asp Gln Thr His Gly Phe Ala Ser Glu Ala Ala Ala Glu
    50                  55                  60

Glu Glu Ala Thr Gln Arg Leu Ala Arg Leu Arg Thr Leu Thr Leu Ala
65                  70                  75                  80

Ala Phe Glu Gly Ala Glu Met Gln Asp Pro Ala Phe Ala Ala Phe Gln
                85                  90                  95

Glu Val Ala Leu Thr His Gly Ile Thr Pro Arg Met Ala Leu Asp His
            100                 105                 110

Leu Asp Gly Phe Ala Met Asp Val Ala Gln Thr Arg Tyr Val Thr Phe
        115                 120                 125

Glu Asp Thr Leu Arg Tyr Cys Tyr His Val Ala Gly Val Val Gly Leu
    130                 135                 140

Met Met Ala Arg Val Met Gly Val Arg Asp Glu Arg Val Leu Asp Arg
145                 150                 155                 160

Ala Cys Asp Leu Gly Leu Ala Phe Gln Leu Thr Asn Ile Ala Arg Asp
                165                 170                 175

Ile Ile Asp Asp Ala Ala Ile Asp Arg Cys Tyr Leu Pro Ala Glu Trp
            180                 185                 190

Leu Gln Asp Ala Gly Leu Thr Pro Glu Asn Tyr Ala Ala Arg Glu Asn
        195                 200                 205

Arg Pro Ala Leu Ala Arg Val Ala Glu Arg Leu Ile Asp Ala Ala Glu
    210                 215                 220

Pro Tyr Tyr Ile Ser Ser Gln Ala Gly Leu His Asp Leu Pro Pro Arg
225                 230                 235                 240

Cys Ala Trp Ala Ile Ala Thr Ala Arg Ser Val Tyr Arg Glu Ile Gly
                245                 250                 255

Ile Lys Val Lys Ala Ala Gly Gly Ser Ala Trp Asp Arg Arg Gln His
            260                 265                 270

Thr Ser Lys Gly Glu Lys Ile Ala Met Leu Met Ala Ala Pro Gly Gln
        275                 280                 285

Val Ile Arg Ala Lys Thr Thr Arg Val Thr Pro Arg Pro Ala Gly Leu
    290                 295                 300

Trp Gln Arg Pro Val  *
```

305 310

(2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 171 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: unknown
(D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: DNA (genomic)

(ix) FEATURE:
(A) NAME/KEY: CDS
(B) LOCATION: 1..171

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

```
ATG GCT TCC TCA GTT CTT TCC TCT GCA GCA GTT GCC ACC CGC AGC AAT
                                                              48
Met Ala Ser Ser Val Leu Ser Ser Ala Ala Val Ala Thr Arg Ser Asn
 1               5                  10                  15

GTT GCT CAA GCT AAC ATG GTG GCG CCT TTC ACT GGC CTT AAG TCA GCT
                                                              96
Val Ala Gln Ala Asn Met Val Ala Pro Phe Thr Gly Leu Lys Ser Ala
            20                  25                  30

GCC TCA TTC CCT GTT TCA AGG AAG CAA AAC CTT GAC ATC ACT TCC ATT
                                                             144
Ala Ser Phe Pro Val Ser Arg Lys Gln Asn Leu Asp Ile Thr Ser Ile
        35                  40                  45

GCC AGC AAC GGC GGA AGA GTG CAA TGC
                                    171
Ala Ser Asn Gly Gly Arg Val Gln Cys
    50                  55
```

(2) INFORMATION FOR SEQ ID NO:5:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 57 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

```
Met Ala Ser Ser Val Leu Ser Ser Ala Ala Val Ala Thr Arg Ser Asn
 1               5                  10                  15

Val Ala Gln Ala Asn Met Val Ala Pro Phe Thr Gly Leu Lys Ser Ala
            20                  25                  30

Ala Ser Phe Pro Val Ser Arg Lys Gln Asn Leu Asp Ile Thr Ser Ile
        35                  40                  45
```

Ala Ser Asn Gly Gly Arg Val Gln Cys
50 55

(2) INFORMATION FOR SEQ ID NO:6:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 263 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: unknown
(D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

GAATTTCCCC GATCGTTCAA ACATTTGGCA ATAAAGTTTC TTAAGATTGA ATCCTGTTGC 60

CGGTCTTGCG ATGATTATCA TATAATTTCT GTTGAATTAC GTTAAGCATG TAATAATTAA 120

CATGTAATGC ATGACGTTAT TTATGAGATG GGTTTTTATG ATTAGAGTCC CGCAATTATA 180

CATTTAATAC GCGATAGAAA ACAAAATATA GCGCGCAAAC TAGGATAAAT TATCGCGCGC 240

GGTGTCATCT ATGTTACTAG ATC 263

(2) INFORMATION FOR SEQ ID NO:7:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: unknown
(D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

NNGAATTCAT TTTTGACATC
20

(2) INFORMATION FOR SEQ ID NO:8:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: unknown
(D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

GCTTTCCATG GTCTTTTGCA
20

CLAIMS

1. A method for visually identifying transgenic plant cells or tissues from non-transgenic plant cells or tissues by carotenoid pigmentation, the method comprising the steps of:

a) culturing transgenic plant and non-transgenic plant cells or tissues in a culture medium, wherein said transgenic cells or tissues contain a heterologous, recombinant chimeric DNA segment containing at least one expression cassette provided that at least one expression cassette comprises:

(1) a promoter DNA segment which functions in plant cells or tissues;

(2) a DNA segment comprising a plastid targeting signal fused to the coding region of the phytoene synthase gene of the *Erwinia* genus group of genes which express a colored member of the biosynthetic pathway, wherein said DNA segment produces RNA which encodes a chimeric polypeptide and said polypeptide results in the production of carotenoid pigmentation in plant cells or tissues; and

(3) a 3' non-translated DNA segment which terminates transcription and adds a polyadenylated tract of residues to the 3' end of the RNA segment;

b) growing said transgenic and non-transgenic cells or tissues for a sufficient amount of time in said medium to allow the transgenic cells or tissues to express the phytoene synthase gene; and

c) identifying the transgenic cells or tissues by their carotenoid pigmentation.

2. A method for visually identifying transgenic plant cells or tissues from non-transgenic plant cells or tissues by carotenoid pigmentation, the method comprising the steps of:

a) culturing transgenic and non-transgenic plant

cells or tissues in a culture medium, wherein said transgenic plant cells or tissues contain a heterologous, recombinant chimeric DNA segment containing at least two expression cassettes, wherein the first expression cassette comprises:

(1) a promoter DNA segment which functions in plant cells or tissues;

(2) a DNA segment comprising a plastid targeting signal fused to the coding region of the phytoene synthase gene of the *Erwinia* genus group of genes which express a colored member of the biosynthetic pathway, wherein said DNA segment produces RNA which encodes a chimeric polypeptide and said polypeptide results in the production of carotenoid pigmentation in plant cells or tissues; and

(3) a 3' non-translated DNA segment which terminates transcription and adds a polyadenylated tract of residues to the 3' end of the RNA segment;

and the second expression cassette comprises:

(1) a promoter DNA segment which functions in plant cells or tissues;

(2) a DNA segment which encodes a second gene which produces RNA which encodes a target protein; and

(3) a 3' non-translated DNA segment which terminates transcription and adds a polyadenylated tract of residues to the 3' end of the RNA segment;

b) growing said transgenic and non-transgenic cells or tissues for a sufficient amount of time in said medium to allow the transformed cells or tissues to express the phytoene synthase gene; and

c) identifying the transformed cells or tissues by their carotenoid pigmentation.

3. The method of claims 1 or 2 wherein the *Erwinia* genus is selected from the group consisting of: *Erwinia*

*uredovora* and *Erwinia herbicola*.

4. A method for visually identifying transgenic plant cells or tissues from non-transgenic plant cells or tissues by carotenoid pigmentation, the method comprising the steps of:

a) culturing transgenic and non-transgenic plant cells or tissues in a culture medium, wherein said transgenic plant cells or tissues contain a heterologous, recombinant chimeric DNA segment containing at least one expression cassette provided that at least one expression cassette comprises:

(1) a promoter DNA segment which functions in plant cells or tissues;

(2) a DNA segment comprising a plastid targeting signal fused to the coding region of the phytoene synthase gene of the *Erwinia* genus group from *Erwinia herbicola*, wherein said DNA segment produces RNA which encodes a chimeric polypeptide and said polypeptide results in the production of carotenoid pigmentation in plant cells or tissues; and

(3) a 3' non-translated DNA segment which terminates transcription and adds a polyadenylated tract of residues to the 3' end of the RNA segment;

b) growing said transgenic and non-transgenic cells or tissues for a sufficient amount of time in said medium to allow the transformed cells to express the phytoene synthase gene; and

c) identifying the transgenic cells or tissues by their carotenoid pigmentation.

5. A method for visually identifying transgenic plant cells or tissues from non-transgenic plant cells or tissues by carotenoid pigmentation, the method comprising the steps of:

a) culturing transgenic and transgenic plant cells or

tissues in a culture medium, wherein said transgenic plant cells or tissues contain a heterologous, recombinant chimeric DNA segment containing at least two expression cassettes, wherein the first expression cassette comprises:

(1) a promoter DNA segment which functions in plant cells or tissues;

(2) a DNA segment comprising a plastid targeting signal fused to the coding region of the phytoene synthase gene from *Erwinia herbicola*, wherein said DNA segment produces RNA which encodes a chimeric polypeptide and said polypeptide results in the production of carotenoid pigmentation in plant cells or tissues; and

(3) a 3' non-translated DNA segment which terminates transcription and adds a polyadenylated tract of residues to the 3' end of the RNA segment;

and the second expression cassette comprises:

(1) a promoter DNA segment which functions in plant cells or tissues;

(2) a DNA segment which encodes a second gene which produces RNA which encodes a target protein; and

(3) a 3' non-translated DNA segment which terminates transcription and adds a polyadenylated tract of residues to the 3' end of the RNA segment;

b) growing said transgenic and non-transgenic cells or tissues for a sufficient amount of time in said medium to allow the transgenic cells or tissues to express the phytoene synthase gene; and

c) identifying the transformed cells or tissues by their carotenoid pigmentation.

6. The method of claims 1, 2, 4 or 5 wherein the recombinant DNA sequence is introduced into the plant cells or tissues by microprojectile bombardment.

7. The method of claims 1, 2, 4 or 5 wherein the

recombinant DNA sequence is introduced into the plant cells or tissues by microinjection.

8. The method of claims 1, 2, 4 or 5 wherein the recombinant DNA sequence is introduced into a vector.

9. The method of claim 8 wherein the vector is a binary vector in an appropriate strain of *Agrobacterium tumefaciens*.

10. The method of claim 8 wherein the vector is a cointegrating vector.

11. The method of claim 9 wherein the binary vector from *Agrobacterium tumefaciens* is introduced into the plant cells via *Agrobacterium*-mediated gene transfer.

12. The method of claims 1, 2, 4 or 5 wherein the plant cells or tissues are selected from the group consisting of tomato, cucurbits, pepper, lettuce and carrots.

13. A method for selectively growing transgenic plants from a mixture of transgenic and non-transgenic plant cells or tissues comprising the steps of:

a) culturing transgenic and non-transgenic plant cells or tissues in a culture medium, wherein said transgenic plant cells or tissues contain a heterologous, recombinant chimeric DNA segment containing at least one expression cassette provided that at least one expression cassette comprises:

(1) a promoter DNA segment which functions in plant cells or tissues;

(2) a DNA segment comprising a plastid targeting signal fused to the coding region of the phytoene synthase gene of the *Erwinia* genus group of genes which express a colored member of the biosynthetic pathway, wherein said DNA segment produces RNA which encodes a chimeric polypeptide and said polypeptide results in the production of carotenoid pigmentation in plant cells or tissues; and

(3) a 3' non-translated DNA segment which terminates transcription and adds a polyadenylated tract of residues to the 3' end of the RNA segment;

b) growing said transgenic and non-transgenic cells or tissues for a sufficient amount of time in said medium to allow the transgenic cells to or tissues express the phytoene synthase gene; and

c) identifying the transgenic cells or tissues by their carotenoid pigmentation;

d) recovering the transgenic cells or tissues; and

e) regenerating plants from said transgenic cells or tissues.

14. A method for selectively growing transgenic plants from a mixture of transgenic and non-transgenic plant cells or tissues comprising the steps of:

a) culturing transgenic and non-transgenic plant cells or tissues in a culture medium, wherein said transgenic plant cells or tissues contain a heterologous, recombinant chimeric DNA segment containing at least two expression cassettes, wherein the first expression cassette comprises:

(1) a promoter DNA segment which functions in plant cells or tissues;

(2) a DNA segment comprising a plastid targeting signal fused to the coding region of the phytoene synthase gene of the *Erwinia* genus group of genes which express a colored member of the biosynthetic pathway, wherein said DNA segment produces RNA which encodes a chimeric polypeptide and said polypeptide results in the production of carotenoid pigmentation in plant cells or tissues; and

(3) a 3' non-translated DNA segment which terminates transcription and adds a polyadenylated tract of residues to the 3' end of the RNA segment;

and the second expression cassette comprises:

(1) a promoter DNA segment which functions in plant cells or tissues;

(2) a DNA segment which encodes a second gene which produces RNA which encodes a target protein; and

(3) a 3' non-translated DNA segment which terminates transcription and adds a polyadenylated tract of residues to the 3' end of the RNA segment;

b) growing said said transgenic and non-transgenic cells or tissues for a sufficient amount of time in said medium to allow the transgenic cells or tissues to express the phytoene synthase gene; and

c) identifying the transgenic cells or tissues by their carotenoid pigmentation;

d) recovering the transgenic cells or tissues; and

e) regenerating plants from said transgenic cells or tissues.

15. The method of claims 13 or 14 wherein the *Erwinia* genus is selected from the group consisting of: *Erwinia uredovora* and *Erwinia herbicola*.

16. A method for selectively growing transgenic plants from a mixture of transgenic and non-transgenic plant cells or tissues comprising the steps of:

a) culturing transgenic and non-transgenic plant cells or tissues in a culture medium, wherein said transgenic cells or tissues contain a heterologous, recombinant chimeric DNA segment containing at least one expression cassette provided that at least one expression cassette comprises:

(1) a promoter DNA segment which functions in plant cells or tissues;

(2) a DNA segment comprising a plastid targeting signal fused to the coding region of the phytoene synthase gene of the *Erwinia* genus group from *Erwinia herbicola*, wherein said DNA segment produces RNA which encodes a

chimeric polypeptide and said polypeptide results in the production of carotenoid pigmentation in plant cells or tissues; and

(3) a 3' non-translated DNA segment which terminates transcription and adds a polyadenylated tract of residues to the 3' end of the RNA segment;

b) growing said said transgenic and non-transgenic cells or tissues for a sufficient amount of time in said medium to allow the transgenic cells or tissues to express the phytoene synthase gene; and

c) identifying the transgenic cells or tissues by their carotenoid pigmentation;

d) recovering the transgenic cells or tissues; and

e) regenerating plants from said transgenic cells or tissues.

17. A method for selectively growing transgenic plants from a mixture of transgenic and non-transgenic plant cells or tissues comprising the steps of:

a) culturing transgenic and non-transgenic plant cells or tissues in a culture medium, wherein said transgenic cells or tissues contain a heterologous, recombinant chimeric DNA segment containing at least two expression cassettes, wherein the first expression cassette comprises:

(1) a promoter DNA segment which functions in plant cells or tissues;

(2) a DNA segment comprising a plastid targeting signal fused to the coding region of the phytoene synthase gene from *Erwinia herbicola*, wherein said DNA segment produces RNA which encodes a chimeric polypeptide and said polypeptide results in the production of carotenoid pigmentation in plant cells or tissues; and

(3) a 3' non-translated DNA segment which terminates transcription and adds a polyadenylated tract of

residues to the 3' end of the RNA segment;

and the second expression cassette comprises:

(1) a promoter DNA segment which functions in plant cells or tissues;

(2) a DNA segment which encodes a second gene which produces RNA which encodes a target protein; and

(3) a 3' non-translated DNA segment which terminates transcription and adds a polyadenylated tract of residues to the 3' end of the RNA segment;

b) growing said said transgenic and non-transgenic cells or tissues for a sufficient amount of time in said medium to allow the transgenic cells or tissues to express the phytoene synthase gene; and

c) identifying the transgenic cells or tissues by their carotenoid pigmentation;

d) recovering the transgenic cells or tissues; and

e) regenerating plants from said transgenic cells or tissues.

18. The method of claims 13, 14, 16 or 17, wherein the plant is selected from the group consisting of: tomato, cucurbits, pepper, lettuce and carrots.

19. A method for identifying proprietary transgenic plants, the method comprising the steps of:

a) supplying at least one proprietary transgenic plant to be identified;

b) culturing explants from said proprietary plant in a culture medium, wherein said proprietary transgenic plant contains a heterologous, recombinant chimeric DNA segment containing at least one expression cassette provided that at least one expression cassette comprises:

(1) a promoter DNA segment which functions in plant cells or tissues;

(2) a DNA segment comprising a plastid targeting signal fused to the coding region of the phytoene synthase

gene of the *Erwinia* genus group of genes which express a colored member of the biosynthetic pathway, wherein said DNA segment produces RNA which encodes a chimeric polypeptide and said polypeptide results in the production of carotenoid pigmentation in plant cells or tissues; and

(3) a 3' non-translated DNA segment which terminates transcription and adds a polyadenylated tract of residues to the 3' end of the RNA segment;

b) growing said said explants for a sufficient amount of time in said medium to allow the transgenic cells or tissues to express the phytoene synthase gene; and

c) identifying the proprietary transgenic cells or tissues by their carotenoid pigmentation.

20. A method for identifying proprietary transgenic plants, the method comprising the steps of:

a) supplying at least one proprietary transgenic plant to be identified;

b) culturing explants from said proprietary transgenic plant in a culture medium, wherein said proprietary transgenic plant contains a heterologous, recombinant chimeric DNA segment containing at least two expression cassettes, wherein the first expression cassette comprises:

(1) a promoter DNA segment which functions in plant cells or tissues;

(2) a DNA segment comprising a plastid targeting signal fused to the coding region of the phytoene synthase gene of the *Erwinia* genus group of genes which express a colored member of the biosynthetic pathway, wherein said DNA segment produces RNA which encodes a chimeric polypeptide and said polypeptide results in the production of carotenoid pigmentation in plant cells or tissues; and

(3) a 3' non-translated DNA segment which terminates transcription and adds a polyadenylated tract of

residues to the 3' end of the RNA segment;

and the second expression cassette comprises:

(1) a promoter DNA segment which functions in plant cells or tissues;

(2) a DNA segment which encodes a second gene which produces RNA which encodes a target protein; and

(3) a 3' non-translated DNA segment which terminates transcription and adds a polyadenylated tract of residues to the 3' end of the RNA segment;

b) growing said said explants for a sufficient amount of time in said medium to allow the transgenic cells or tissues to express the phytoene synthase gene; and

c) identifying the proprietary transgenic cells by their carotenoid pigmentation.

21. The method of claims 19 or 20 wherein the *Erwinia* genus is selected from the group consisting of: *Erwinia uredovora* and *Erwinia herbicola*.

22. A method for identifying proprietary transgenic plants, the method comprising the steps of:

a) supplying at least one proprietary transgenic plant to be identified;

b) culturing explants from said proprietary plant in a culture medium, wherein said proprietary transgenic plant contains a heterologous, recombinant chimeric DNA segment containing at least one expression cassette provided that at least one expression cassette comprises:

(1) a promoter DNA segment which functions in plant cells or tissues;

(2) a DNA segment comprising a plastid targeting signal fused to the coding region of the phytoene synthase gene of the *Erwinia* genus group from *Erwinia herbicola*, wherein said DNA segment produces RNA which encodes a chimeric polypeptide and said polypeptide results in the production of carotenoid pigmentation in plant cells and

tissues; and

(3) a 3' non-translated DNA segment which terminates transcription and adds a polyadenylated tract of residues to the 3' end of the RNA segment;

b) growing said said transgenic and non-transgenic cells or tissues for a sufficient amount of time in said medium to allow the transgenic cells to express the phytoene synthase gene; and

c) identifying the proprietary transgenic cells oir tissues by their carotenoid pigmentation.

23. A method for identifying proprietary transgenic plants, the method comprising the steps of:

a) supplying at least one proprietary transgenic plant to be identified;

b) culturing explants from said proprietary plant in a culture medium, wherein said proprietary transgenic plant contains a heterologous, recombinant chimeric DNA segment containing at least two expression cassettes, wherein the first expression cassette comprises:

(1) a promoter DNA segment which functions in plant cells or tissues;

(2) a DNA segment comprising a plastid targeting signal fused to the coding region of the phytoene synthase gene from *Erwinia herbicola*, wherein said DNA segment produces RNA which encodes a chimeric polypeptide and said polypeptide results in the production of carotenoid pigmentation in plant cells or tissues; and

(3) a 3' non-translated DNA segment which terminates transcription and adds a polyadenylated tract of residues to the 3' end of the RNA segment;

and the second expression cassette comprises:

(1) a promoter DNA segment which functions in plant cells or tissues;

(2) a DNA segment which encodes a second gene

which produces RNA which encodes a target protein; and

(3) a 3' non-translated DNA segment which terminates transcription and adds a polyadenylated tract of residues to the 3' end of the RNA segment;

b) growing said said transgenic and non-transgenic cells or tissues for a sufficient amount of time in said medium to allow the transgenic cells to express the phytoene synthase gene; and

c) identifying the proprietary transgenic cells or tissues by their carotenoid pigmentation.

24. The method of claims 19, 20, 22 and 23, wherein the plants are selected from the group consisting of: tomato, cucurbits, pepper, lettuce and carrots.

25. A transgenic plant whose genome comprises a heterologous chimeric DNA segment that contains at least one expression cassette provided that at least one expression cassette comprises:

(1) a promoter DNA segment which functions in plant cells or tissues;

(2) a DNA segment comprising a plastid targeting signal
fused to the coding region of the phytoene synthase gene of the *Erwinia* genus group of genes which express a colored member of the biosynthetic pathway, wherein said DNA segment produces RNA
which encodes a chimeric polypeptide and said polypeptide results in the production of carotenoid pigmentation in plant cells or tissues; and

(3) a 3' non-translated DNA segment which terminates transcription and adds a polyadenylated tract of residues to the 3' end of the RNA segment.

26. A transgenic plant whose genome comprises a heterologous DNA segment that contains at least two

expression cassettes wherein the first expression cassette comprises:

(1) a promoter DNA segment which functions in plant cells or tissues;

(2) a DNA segment comprising a plastid targeting signal fused to the coding region of the phytoene synthase gene of the *Erwinia* genus group of genes which express a colored member of the biosynthetic pathway, wherein said DNA segment produces RNA which encodes a chimeric polypeptide and said polypeptide results in the production of carotenoid pigmentation in plant cells or tissues; and

(3) a 3' non-translated DNA segment which terminates transcription and adds a polyadenylated tract of residues to the 3' end of the RNA segment;

and the second expression cassette comprises:

(1) a promoter DNA segment which functions in plant cells or tissues;

(2) a DNA segment which encodes a second gene which produces RNA which encodes a target protein; and

(3) a 3' non-translated DNA segment which terminates transcription and adds a polyadenylated tract of residues to the 3' end of the RNA segment.

27. The method of claims 25 or 26 wherein the *Erwinia* genus is selected from the group consisting of: *Erwinia uredovora* and *Erwinia herbicola*.

28. A transgenic plant whose genome comprises a heterologous chimeric DNA segment that contains at least one expression cassette provided that at least one expression cassette comprises:

(1) a promoter DNA segment which functions in plant cells or tissues;

(2) a DNA segment comprising a plastid targeting signal fused to the coding region of the phytoene synthase gene of the *Erwinia* genus group of *Erwinia herbicola*

wherein said DNA segment produces RNA which encodes a chimeric polypeptide and said polypeptide results in the production of carotenoid pigmentation in plant cells or tissues; and

(3) a 3' non-translated DNA segment which terminates transcription and adds a polyadenylated tract of residues to the 3' end of the RNA segment.

29. A transgenic plant whose genome comprises a heterologous chimeric DNA segment that contains at least two expression cassettes wherein the first expression cassette comprises:

(1) a promoter DNA segment which functions in plant cells or tissues;

(2) a DNA segment comprising a plastid targeting signal
fused to the coding region of the phytoene synthase gene of the *Erwinia* genus group of *Erwinia herbicola*, wherein said DNA segment produces RNA which encodes a chimeric polypeptide and said polypeptide results in the production of carotenoid pigmentation in plant cells; and

(3) a 3' non-translated DNA segment which terminates transcription and adds a polyadenylated tract of residues to the 3; end of the RNA segment;

and the second expression cassette comprises:

(1) a promoter DNA segment which functions in plant
cells;

(2) a DNA segment which encodes a second gene which produces RNA which encodes a target protein; and

(3) a 3' non-translated DNA segment which terminates transcription and adds a polyadenylated tract of residues to the 3' end of the RNA segment.

30. The plants of claims 25, 26, 28 or 29 wherein the plant is selected from the group consisting of: tomato,

cucurbits, pepper, lettuce and carrots.

31. Seed from a transgenic plant according to claims 25, 26, 28 or 29.

32. A plasmid designated pETO203 having American Type Culture Collection accession number 97282.

1/3

FIGURE 1

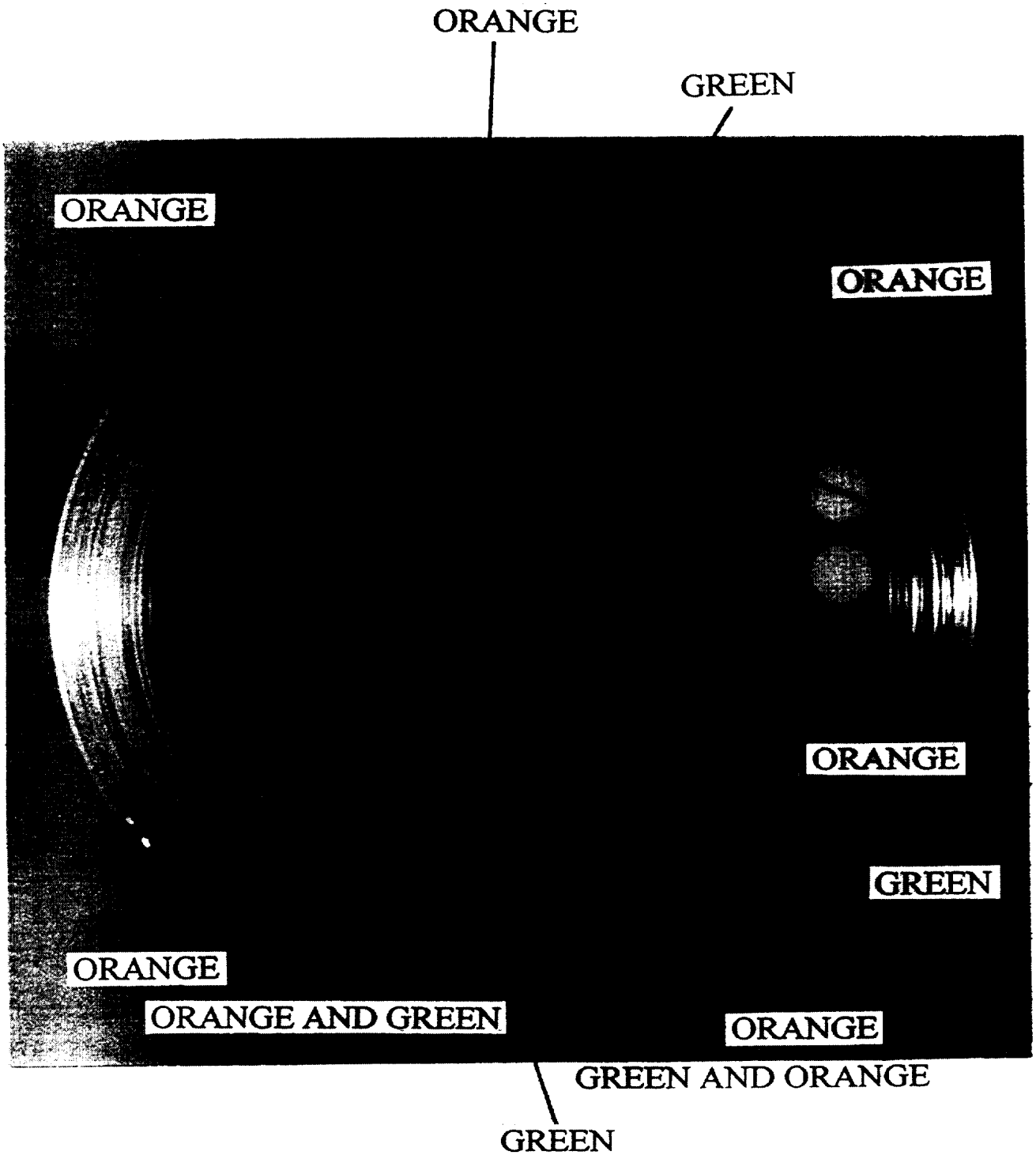

ORANGE
GREEN
ORANGE
ORANGE
ORANGE
GREEN
ORANGE
ORANGE AND GREEN
ORANGE
GREEN AND ORANGE
GREEN

FIGURE 2

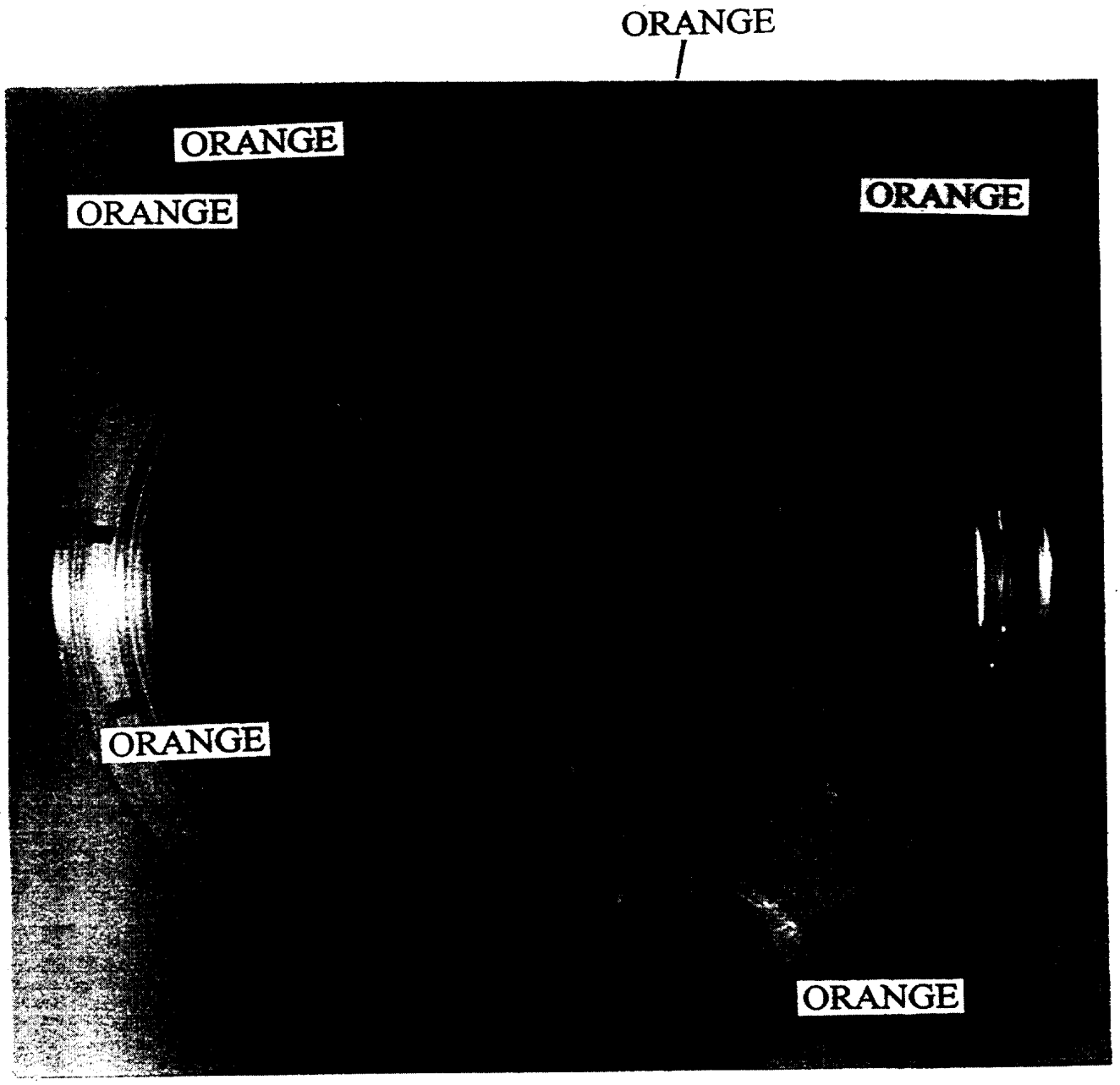
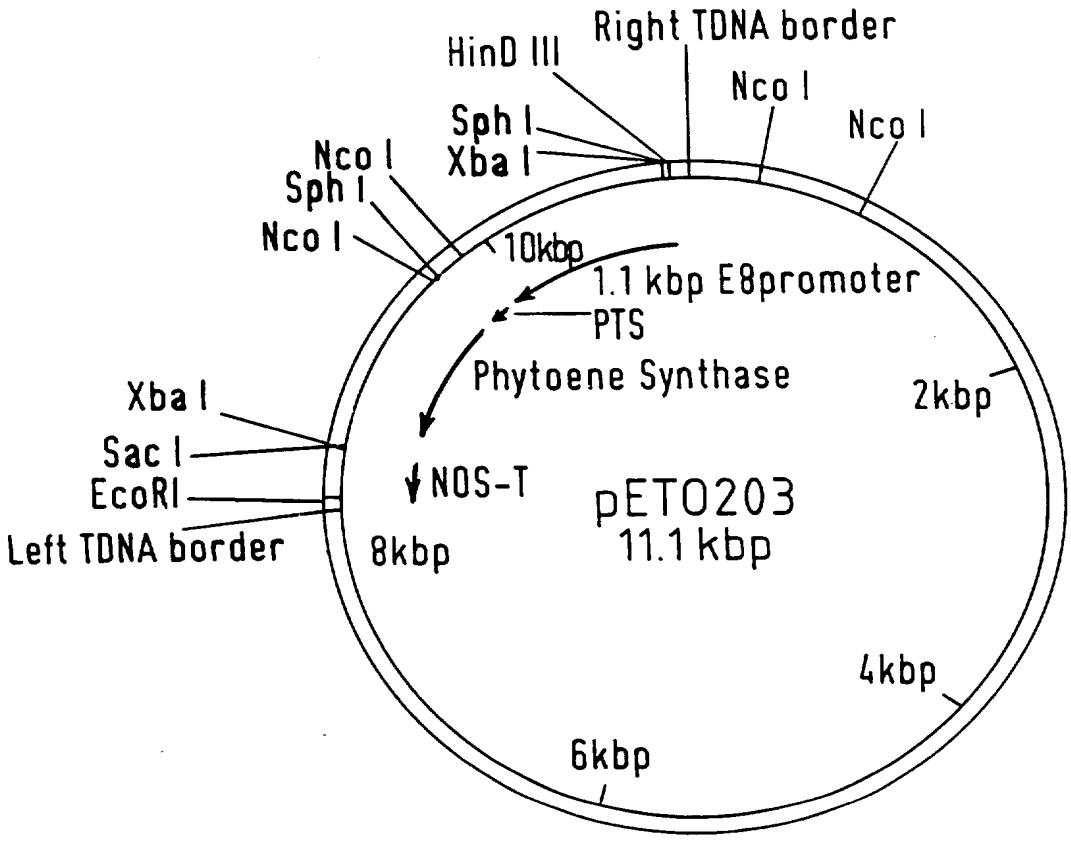


FIGURE 3


Right TDNA border
HinD III
Nco I
Sph I
Xba I
Nco I
Nco I
Sph I
Nco I
10kbp
1.1 kbp E8promoter
PTS
Phytoene Synthase
2kbp
Xba I
Sac I
EcoRI
Left TDNA border
NOS-T
pETO203
11.1 kbp
8kbp
4kbp
6kbp

# INTERNATIONAL SEARCH REPORT

Inte onal Application No
PCT/US 96/04313

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C12N15/82 A01H5/00

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C12N A01H

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

| Category * | Citation of document, with indication, where appropriate, of the relevant passages | Relevant to claim No. |
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| A | THE PLANT JOURNAL,<br>vol. 6, no. 4, 1994,<br>pages 481-489, XP002012919<br>MISAWA, N., ET AL.: "Expression of an Erwinia phytoene desaturase gene not only confers multiple resistance to herbicides interfering with carotenoid biosynthesis but also alters xanthophyll metabolism in transgenic plants"<br>see the whole document | 1-32 |

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[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

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"A" document defining the general state of the art which is not considered to be of particular relevance

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| Date of the actual completion of the international search | Date of mailing of the international search report |
|---|---|
| 21 October 1996 | 28.10.96 |

| Name and mailing address of the ISA | Authorized officer |
|---|---|
| European Patent Office, P.B. 5818 Patentlaan 2<br>NL - 2280 HV Rijswijk<br>Tel. ( + 31-70) 340-2040, Tx. 31 651 epo nl,<br>Fax ( + 31-70) 340-3016 | Maddox, A |

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| E | WO,A,96 13149 (AMOCO CORP) 9 May 1996<br>see page 68, line 25 - page 69, line 14; claims 1-39 | 1-5,8,9, 11,13-31 |

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PCT/US 96/04313

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